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NHEJ protects mycobacteria in stationary phase against the harmful effects of desiccation

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Abstract:

The physiological role of the non homologous end-joining (NHEJ) pathway in the repair of DNA double strand breaks (DSBs) was examined in *Mycobacterium smegmatis* using DNA repair mutants ($\Delta recA$, Δku , $\Delta ligD$, $\Delta ku/ligD$, $\Delta recA/ku/ligD$). Wild-type and mutant strains were exposed to a range of doses of ionizing radiation at specific points in their life-cycle. NHEJ mutant strains (Δku , $\Delta ligD$, $\Delta ku/ligD$) were significantly more sensitive to ionizing radiation (IR) during stationary phase than wild-type *M. smegmatis*. However, there was little difference in IR sensitivity between NHEJ-mutant and wild-type strains in logarithmic phase. Similarly, NHEJ-mutant strains were more sensitive to prolonged desiccation than wild-type *M. smegmatis*. A $\Delta recA$ mutant strain was more sensitive to desiccation and IR during both stationary and especially in logarithmic phase, compared to wild-type strain, but it was significantly less sensitive to IR than the $\Delta recA/ku/ligD$ triple mutant during stationary phase. These data suggest that NHEJ and homologous recombination are the preferred DSB repair pathways employed by *M. smegmatis* during stationary and logarithmic phases, respectively.

Introduction

DNA double-strand breaks (DSBs) are the most lethal type of lesion that the genome can experience, as even a single unrepaired break can result in cell death. Non homologous end-joining (NHEJ) is an essential pathway responsible for the repair of DSBs in higher eukaryotes (1) but was considered to be absent from prokaryotes, which were thought to rely solely on homologous recombination (HR) for the repair of DSBs. More recently, it has been established that many prokaryotic species also possess a functional NHEJ repair complex, consisting of a Ku homologue that binds to the DNA ends and subsequently, recruits a multifunctional DNA ligase (LigD) that covalently joins together the DSB (2,3). Since many bacteria that possess a NHEJ repair apparatus (Ku and LigD) spend much of their life-cycle in stationary phase, it has been speculated that bacteria may rely on NHEJ during states of prolonged mitotic exit (2,4) where the absence of a homologous donor precludes DSB repair by HR, in an analogous manner to G₁ and non-cycling eukaryotic cells. We previously reported that *Bacillus subtilis* strains lacking the NHEJ proteins, Ku (YkoV) and LigD (YkoU), are mildly sensitive to ionizing radiation (IR) (2) suggesting that bacterial NHEJ plays a role in the repair of DSBs *in vivo*. However, the survival rate of NHEJ-mutant strains following IR treatment was much higher than anticipated (2), when compared to NHEJ-deficient eukaryotic cells, suggesting that the bacterial NHEJ repair pathway was less important than expected. Therefore, we sought to address if the NHEJ repair pathway was essential for the repair of DSBs, during any particular stage of the bacterial cell-cycle, and also to discover what type of environmental stress induces physiologically-relevant DSBs that are repaired by NHEJ.

2 Materials and methods

2.1 Bacterial strains

NHEJ-deficient *M. smegmatis* strains used in this work were as previously described by Korycka-Machala *et al.* (5).

2.2 Desiccation survival assays

Desiccation experiments were carried out essentially as previously described (6). Cultures of wild-type and null mutant strains grown from single colonies at 37 °C with shaking in Middlebrook 7H9 broth (Difco) supplemented with 7H9 ADC enrichment media (Difco) were grown to logarithmic phase and collected by centrifugation. They were then washed in four volumes of 10 mM MgSO₄, and resuspended in 5 ml of 10 mM MgSO₄. An aliquot (100 µl) of this suspension was placed upon a sterile glass coverslip, positioned inside a sterile petri dish, and dried at 25 °C in a desiccator containing CaSO₄ with indicator (Drierite) for 21 days. After three weeks the samples were rehydrated by the addition of 1 ml of 10 mM MgSO₄. Each sample was then serially diluted and plated onto 7H10 Middlebrook agar (Difco) supplemented with OADC (Difco) and incubated at 37 °C for three days before colonies were counted. At least three independent experiments were performed and the data averaged. The survival percentage for each strain was calculated based upon colonies present before desiccation.

2.3 IR survival Assays

Cultures of each strain were grown from single colonies at 37 °C with shaking in Middlebrook 7H9 broth (Difco) supplemented with 7H9 ADC enrichment media (Difco).

Aliquots of wild-type and mutant strains were taken from cultures at various points in their growth-curve as indicated in the appropriate figure legends. These aliquots were irradiated using a ^{137}Cs γ -ray source at a dose rate of 0.125 Gy s^{-1} . Radiosensitivity was assessed by a colony survival assay following 0, 75, 225, 450 and 675 Gy irradiation. Aliquots of neat, 10x and 1000x dilutions were spotted onto 7H10 Middlebrook agar (Difco) supplemented with OADC (Difco) and incubated at 37°C for three days before colonies were examined. At least three independent experiments were performed. To produce survival curves, three individual aliquots of each strain were exposed to IR, at the appropriate dosage (0, 225 450 or 675 Gy). Cell suspensions were serially diluted and plated onto 7H10 Middlebrook agar (Difco) supplemented with OADC (Difco) and incubated at 37°C for three days before colonies were counted. Survival percentage at each IR dose and for each strain was calculated based upon the numbers of colonies present before irradiation.

3. Results and Discussion

3.1 NHEJ-deficient strains of *M. smegmatis* are sensitive to IR during stationary phase

To test the possibility that NHEJ protects mycobacteria against the deleterious effects of IR-induced damage, we utilized *M. smegmatis* strains deficient in the NHEJ repair proteins, Ku (Δku), ligase D ($\Delta ligD$) or both ($\Delta ku/ligD$) (5). Bacterial cells isolated at one of four stages of the growth cycle (early, medium, late or stationary phase; Fig. 1, right panel) were irradiated with IR (0-675 Gy) and the cells subsequently plated. The need for multiple time-points reflects the fact that it is not possible to synchronize *M. smegmatis* cells at a particular point in their growth-cycle, instead different sub-populations are

present within a cell suspension in varying proportions through-out (7), in contrast to the dormant state observed in *Bacillus subtilis* endospores in which cell division ceases completely (8). Consequently, any observed phenotype in *M. smegmatis* will likely resemble a trend rather than a distinct requirement.

Figure 1 shows the phenotypic consequences of exposing NHEJ-deletion strains to γ -radiation. Consistent with previous observations (5,9,10), in the absence of DNA damaging agents there was no apparent difference in the growth rates between wild-type *M. smegmatis* and NHEJ-mutant strains. Indeed the colony forming ability of each strain was indistinguishable in both early logarithmic and stationary phase (data not shown); Apparently, Ku and LigD are dispensable for growth under normal conditions. During early exponential growth, both wild-type and the NHEJ-mutant strains showed a similar level of sensitivity to IR, suggesting that NHEJ is not important for DSB repair in rapidly dividing cells (Fig. 1, left panel). However, in late and especially stationary phase, NHEJ-deficient strains were significantly more sensitive to DSBs, the major cytotoxic lesion induced by IR. This experiment was repeated on three independent cultures of each strain and the results were fully reproducible (data not shown). To further characterize the IR sensitivity of the NHEJ-mutants, survival curves were produced by counting colonies on plates from appropriate 10-fold serial dilutions after IR exposure (Fig. 2) during early logarithmic and stationary phase (corresponding to time-points A and D, respectively, right panel of Fig. 1). In line with the survival spot test, there was less than 10-fold difference in viability between any of the strains when irradiated (either 450 or 675 Gy) in early logarithmic phase (Fig. 2A). However, irradiation of the NHEJ-mutant strains in

stationary phase caused a large decrease in viability compared to the wild-type (Fig. 2B). Irradiation (675 Gy) of stationary-phase Δku and $\Delta ku/ligD$ strains caused an approximately 1000-fold decrease in viability, while irradiated $\Delta ligD$ cells were about 100-fold less viable when any were compared to wild-type. The 10-fold decrease in viability of Δku or $\Delta ku/ligD$ cells compared to $\Delta ligD$ cells suggests that while LigD participates exclusively in the same DSB repair pathway as Ku in *M. smegmatis*, Ku may additionally have a role in DSB repair independent of LigD. It should be noted that the *M. smegmatis* genome encodes three ATP dependent DNA ligases (LigB, LigC₁, LigC₂) in addition to LigD and genetic studies suggest a small degree of redundancy between them in regard to recircularisation of transformed linear plasmids, a common NHEJ assay (10,11). Consequently, these other ligases may also contribute to the difference in viability between Δku and $\Delta ligD$ cells after IR exposure.

The role of HR in IR-induced DSB repair was also investigated using RecA mutants. RecA catalyses the pairing of single-stranded DNA with complementary regions of double-stranded DNA during HR. Single ($\Delta recA$), and triple mutant ($\Delta recA/\Delta ku/ligD$) *recA* strains were both viable under normal conditions, consistent with previous results (5), although the $\Delta recA/\Delta ku/ligD$ and $\Delta recA$ mutant strains had an extended, flatter logarithmic phase compared to wild-type (Fig. 3) which made it difficult to directly compare IR sensitivity between these strains during logarithmic phase with wild-type cells. However, the spot test (Fig. 3, left panel) and survival curves (Fig. 4) demonstrated that the $\Delta recA/ku/ligD$ and $\Delta recA$ strains were similarly sensitive to IR during early logarithmic stage (early logarithmic and stationary phases in fig. 4 correspond to time-

points A and D, respectively, in Fig. 3) but that the triple mutant was considerably more sensitive to IR than the $\Delta recA$ strain during stationary stage. Indeed, $\Delta recA$ cells were 1000X less sensitive to IR than $\Delta recA/kuligD$ cells when in stationary phase (Fig. 4B), demonstrating that RecA is non-epistatic with Ku and LigD. Furthermore, the spot test (Fig. 3) also suggested that the $\Delta recA$ strain was less sensitive to IR in stationary phase than during early logarithmic phase. Survival curves (Fig. 4) demonstrated that this was not merely due to reductive cell division increasing cell viability during stationary phase. These results are consistent with a role for Ku and LigD in DSB repair during stationary phase and RecA during logarithmic phase.

3.2 NHEJ pathway protects *M. smegmatis* against the harmful effects of desiccation

Although strains of *M. smegmatis* deficient in NHEJ are sensitive to IR-induced damage, there are no terrestrial environments that produce high doses of this form of radiation; the highest recorded absorbed dose rate is only 175 mGy per year (12). However, it has been reported that ionizing radiation-resistant bacteria can be isolated from natural sources simply by selecting for strains that are highly resistant to desiccation (13), suggesting that IR-resistance of certain bacteria is merely a consequence of the adaptation of many bacterial species to a common physiological stress, namely desiccation (6).

Like, ionising radiation, the cytotoxicity of desiccation derives from the formation of DSBs (6,14,15). A substantial reduction in the molecular weight of *D. radiodurans* chromosomal DNA after exposure to a vacuum has been demonstrated (14). Indeed, the extent of chromosomal fragmentation, adjudged to correspond to at least 60 DSBs, in *D.*

radiodurans following desiccation is comparable to exposure to high doses of IR (6). While, the accumulation of chromosomal DSBs after prolonged desiccation has also been demonstrated in *Escherichia coli* (6).

To test the possibility that NHEJ could protect mycobacteria against the deleterious effects of desiccation-induced DSBs, we again utilized *M. smegmatis* NHEJ-deficient strains (Δku , $\Delta ligD/\Delta ku$, $\Delta ligD$) (5). Logarithmic cells (wild-type and mutants) were spotted onto cover-slips and placed in sterile Petri dishes in desiccators containing a strong desiccating agent ($CaSO_4$) and incubated at 25°C for three weeks. The cells were subsequently recovered by washing the cover-slips followed by appropriate serial dilutions and then plating of the cells (Fig. 5). The survival percentage of each strain was determined by counting colonies before and after desiccation. As illustrated in Fig. 5, a significant number of wild-type *M. smegmatis* colonies grew upon plating of recovered desiccated cells, even after such a prolonged period of desiccation (viability was reduced by approximately 10^4 compared to undesiccated cells). In contrast, cells lacking either one (Δku or $\Delta ligD$) or both of the NHEJ proteins (Δku , $\Delta ligD$) were between 20 and 100-fold less viable than the wild-type strain. The $\Delta recA/\Delta ku/ligD$ and $\Delta recA$ mutants were also significantly less viable than wild-type; about 100- and 20-fold respectively, suggesting that HR is also responsible for DSB repair in rehydrated cells. This may reflect the fact that not all *M. smegmatis* cells enter a non-dividing dormant state during stationary phase (7). Similarly, despite difficulties in direct comparisons, stationary phase wild-type cells were apparently more viable than stationary phase $\Delta recA$ cells after IR exposure. While, there is some evidence of an interplay between HR and NHEJ mediated

DSB repair elicited by the DNA damaging agent mitomycin C in *M. smegmatis* (5). Furthermore, RecA is involved in other cellular pathways in bacteria in addition to HR, such as the SOS response (16), which impacts on several aspects of DNA repair, including NER and BER.

These results suggest that NHEJ-deficient cells accumulate DSBs that, if left unrepaired, are lethal and therefore the prokaryotic NHEJ complex has an important protective role in repairing DSBs produced by the genotoxic effects of desiccation on stationary phase cells and represents the first direct experimental evidence of a physiological role for prokaryotic NHEJ. It also suggests that other bacterial species, that survive for long periods in a prolonged stationary phase, may also utilize NHEJ to protect against the harmful effects of DNA breaks produced by desiccation.

3.3 A role for NHEJ in desiccation resistance in prokaryotes

M. smegmatis, is a unicellular, aerobic, gram-positive bacteria that resides in the soil where it degrades organic matter. *Mycobacteria* are unusual among bacteria as they have a very thick hydrophobic cell wall that reduces desiccation (17); presumably desiccation is environmentally relevant. Like most free-living bacteria, *M. smegmatis* is exposed to environmental conditions (heat and freeze-drying) that can result in prolonged periods of dehydration, resulting in cell desiccation. It is thought that bacterial populations spend most of their time under strong growth limitation in the natural world (18,19) and potentially exist in a non-differentiating state. We propose that many bacterial species

have retained a NHEJ pathway to repair DSBs to ensure continued survival following exposure to genotoxic stress under such limiting conditions.

A functional NHEJ-mediated DSB repair pathway, consisting of LigD and Ku, has recently been identified in *M. tuberculosis* by *in vitro* biochemical techniques (2,3) and plasmid repair assays (9,10). *M. tuberculosis* which causes tuberculosis, resides exclusively within human hosts and depends on transmission between humans for its continued survival (20). Consequently, it is significant that although *M. tuberculosis* is predominantly present in the lungs and airways of infected animals, it has been demonstrated that respiratory droplets, containing bacilli, generated by coughs and sneezes rapidly desiccate, becoming minute particles that remain airborne as ‘droplet nuclei’ (21), potentially carrying infectious bacteria to new hosts. Furthermore, *M. tuberculosis* survives in a non-growing state in the lungs of mice with latent tuberculosis (22). It is under such conditions that these pathogenic bacteria may be exposed to the effects of desiccation and therefore may explain why they have retained a functional NHEJ repair apparatus that has been lost by other species. It is also likely that NHEJ may be important for survival of bacilli within macrophages, where they are continually exposed to other genotoxic stress, such as oxidative damage, that produces DSBs.

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Fig. 1- IR sensitivity of NHEJ-deficient strains of *M. smegmatis*. Aliquots of wild-type and NHEJ-deficient (Δku , $\Delta ligD$ and $\Delta ku/ligD$) strains of *M. smegmatis* were taken from cultures at various points (A-D) in the growth-curve as indicated (right panel). There was essentially no difference in the growth kinetics of the four strains. These aliquots were irradiated using a ^{137}Cs γ -ray source at a dose rate of 0.125 Gy s^{-1} . Radiosensitivity was assessed by a colony survival assay following 0, 75, 225, 450 and 675 Gy of irradiation. Aliquots (10 μl) of neat, 10x and 1000x dilutions of the irradiated cells were spotted onto 7H10 Middlebrook agar (Difco) supplemented with OADC (Difco) and incubated at 37°C for three days before colonies were examined (left panel). This figure is representative of three independent experiments.

Fig. 2 - Survival curve of NHEJ-deficient strains of *M. smegmatis* after IR treatment. Resistance to γ -radiation of wild-type, Δku , $\Delta ligD$ and $\Delta ku/ligD$ *M. smegmatis* strains during early logarithmic phase (**A**) and stationary phase (**B**) was calculated by counting colonies of serially diluted cell cultures following either 0, 450 or 675 Gy irradiation. Each data point is an average of at least three replicate experiments \pm standard deviation. Solid lines indicate the linear regression fits for each data set.

Fig. 3 - IR sensitivity of $\Delta recA$ and $\Delta recA/ku/ligD$ strains of *M. smegmatis*. Aliquots of $\Delta recA$ and $\Delta recA/ku/ligD$ strains of *M. smegmatis* were taken from cultures at various points in the growth-curve as indicated (right panel) and exposed to 0, 75, 225, 450 and 675 Gy of IR. Aliquots (10 μl) of neat, 10x and 1000x dilutions of the irradiated cells

were spotted onto 7H10 Middlebrook agar (Difco) supplemented with OADC (Difco) and incubated at 37° C for four days before colonies were examined (left panel). This figure represents one experiment and is entirely consistent with three other independent experiments.

Fig. 4 - Survival curve of $\Delta recA$ and $\Delta recA/ku/ligD$ strains of *M. smegmatis* after IR treatment. Resistance to γ -irradiation of wild-type, $\Delta recA$ and $\Delta recA/ku/ligD$ *M. smegmatis* strains during early logarithmic phase (**A**) and stationary phase (**B**) was calculated by counting colonies of serially diluted cell cultures following either 0, 225 or 450 Gy irradiation. Each data point is an average of at least three independent experiments \pm standard deviation. Solid lines indicate the linear regression fits.

Fig. 5 - NHEJ-deficient strains of *M. smegmatis* are sensitive to desiccation.

Aliquots of wild-type, Δku , $\Delta ligD$ and $\Delta Ku/ligD$ *M. smegmatis* strains, grown to logarithmic phase, were placed upon a sterile glass cover-slips, positioned inside a sterile petri dish, and dried at 25° C in a desiccator containing CaSO₄ with indicator (Drierite) for 21 days. The cells were subsequently recovered by washing the cover-slips and then plating of the cells (left panel). The percentage survival for each strain was determined by counting colonies (right panel). Each data point represents an average from three independent experiments performed in triplicate \pm standard deviation.

Figure 1

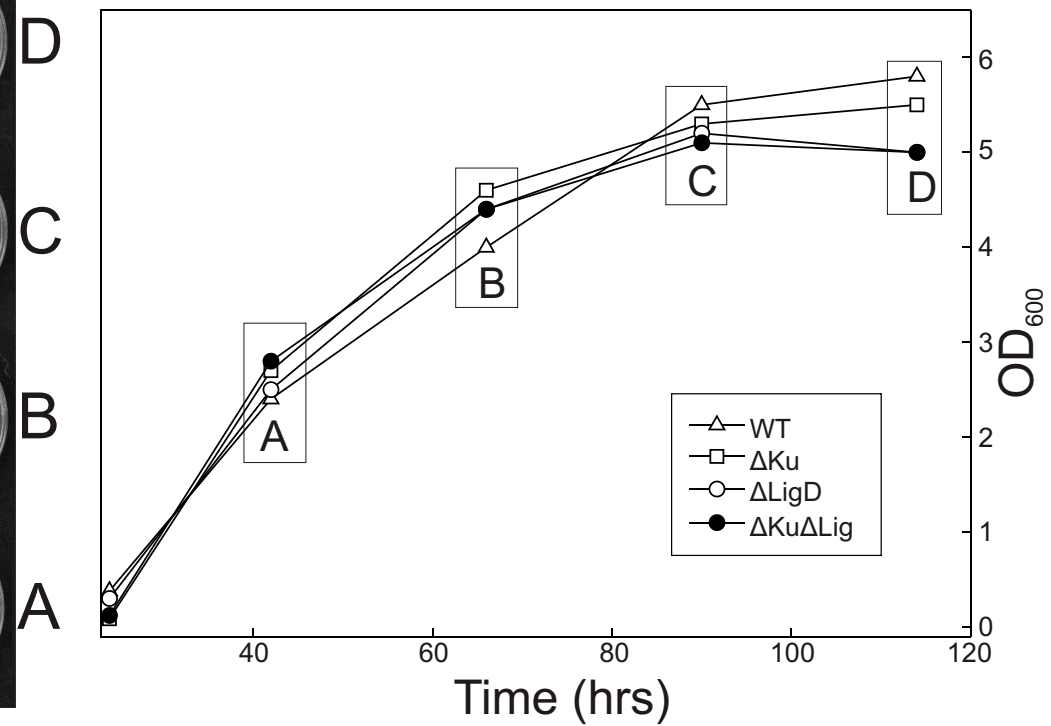
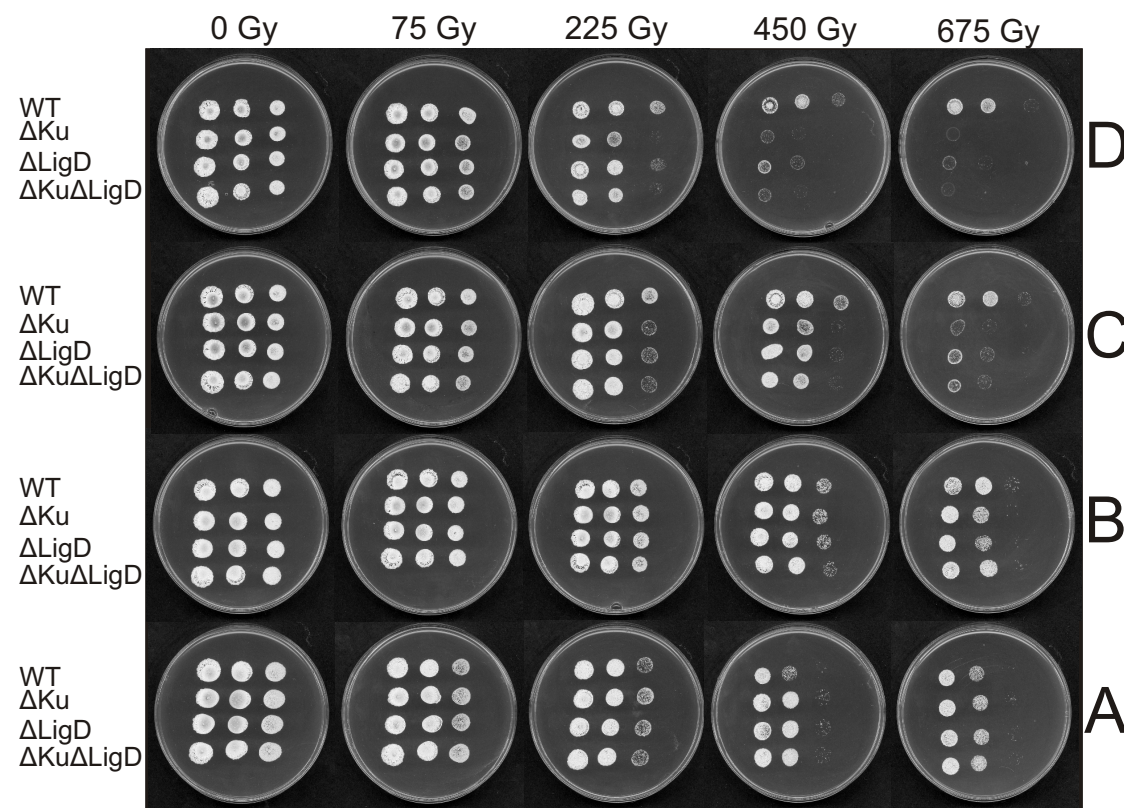
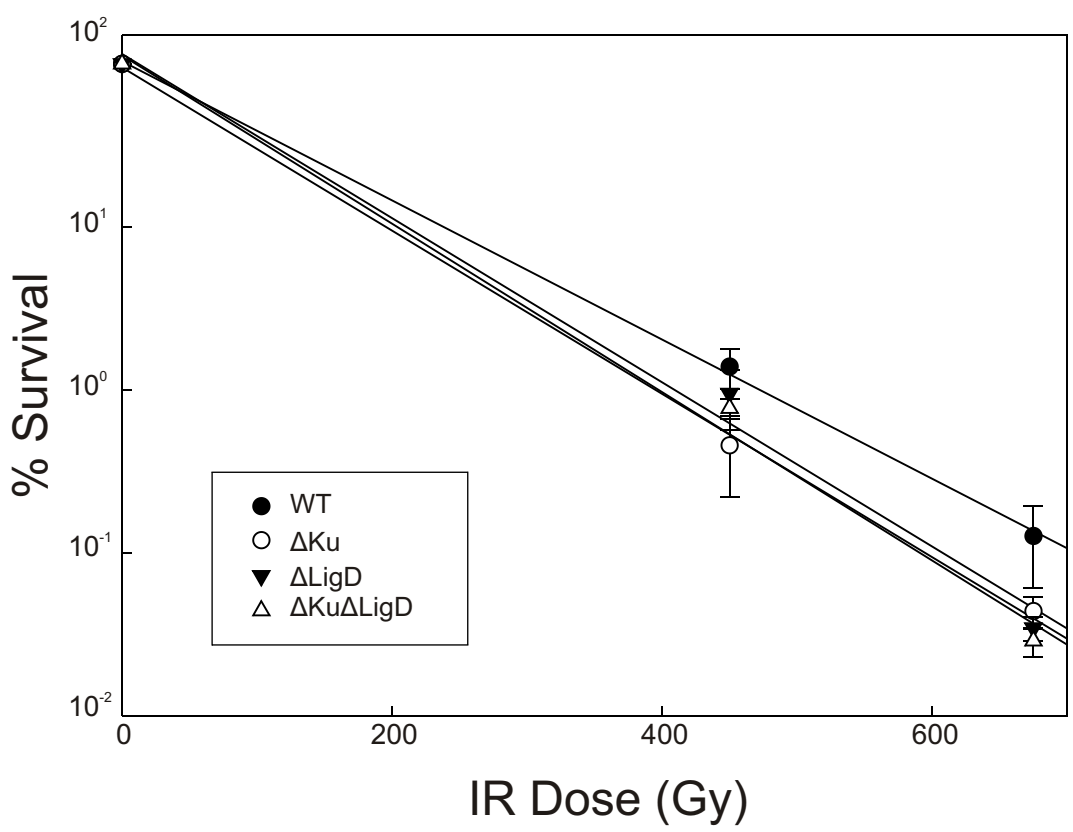


Figure 2

A



B

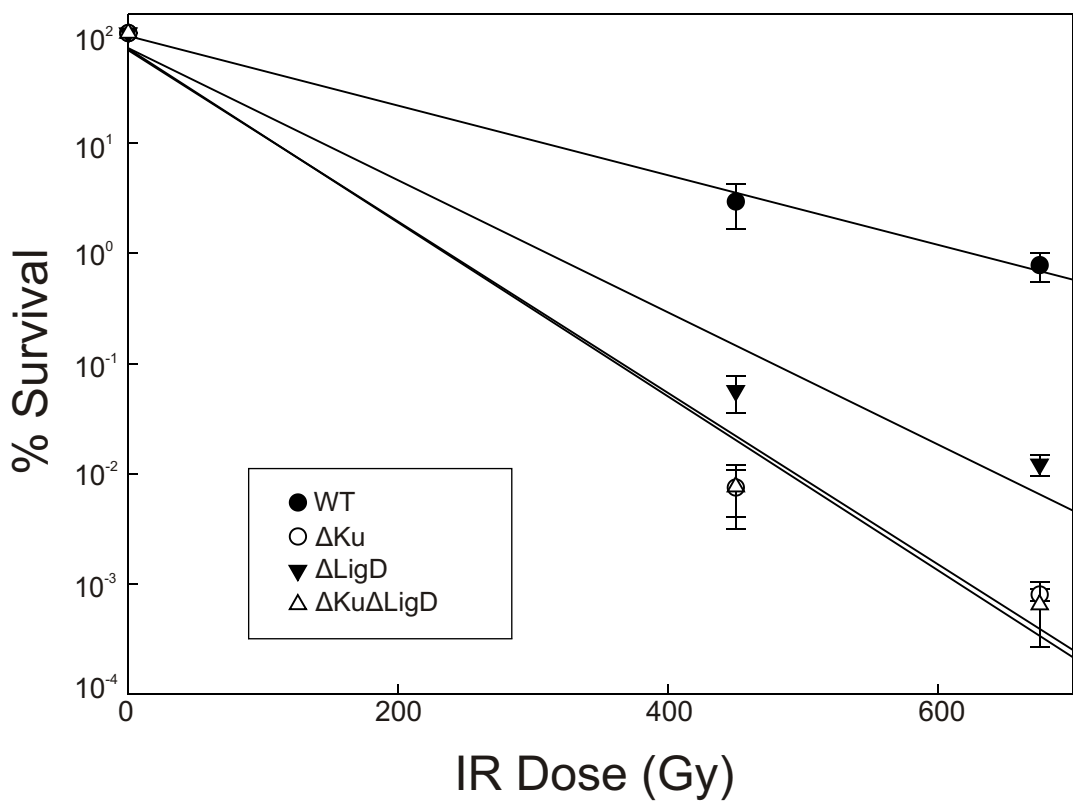


Figure 3

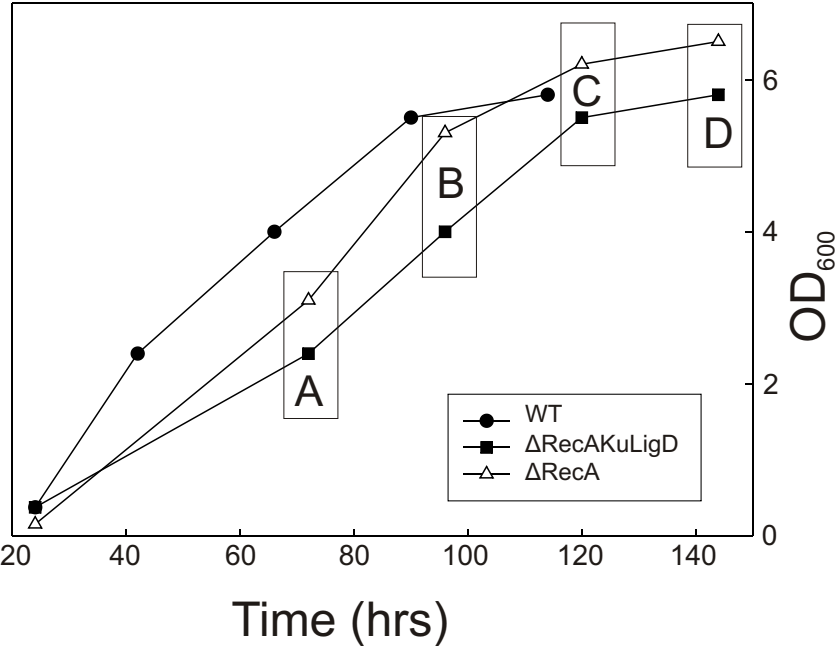
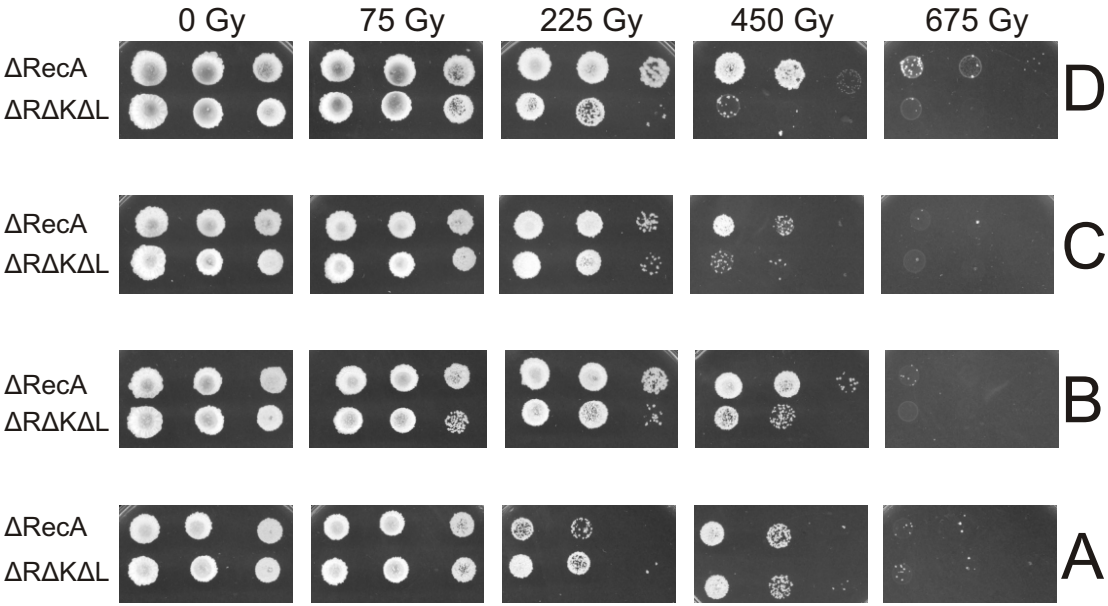
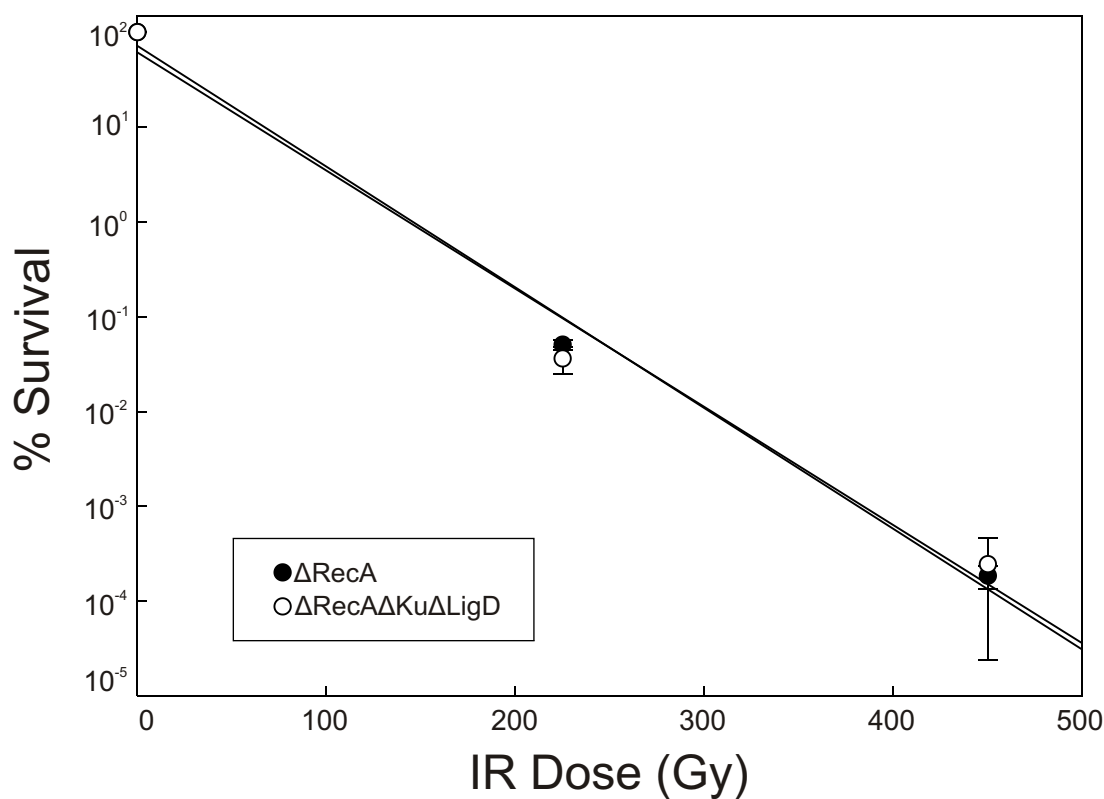


Figure 4

A



B

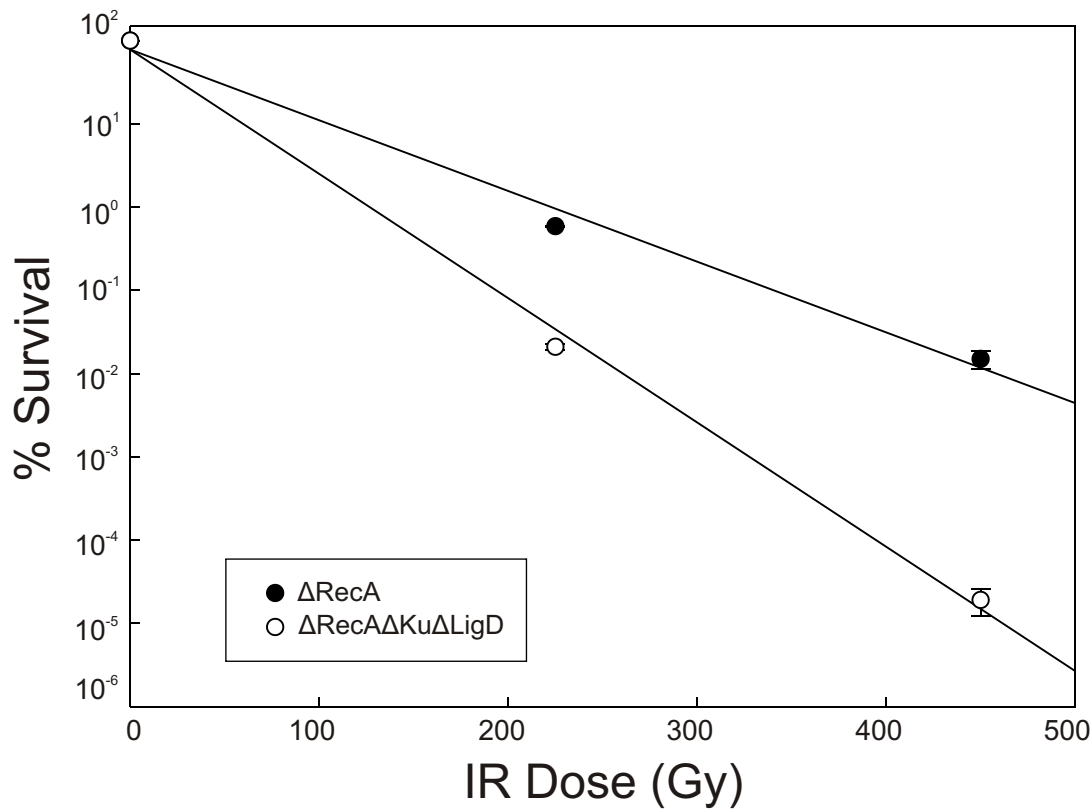
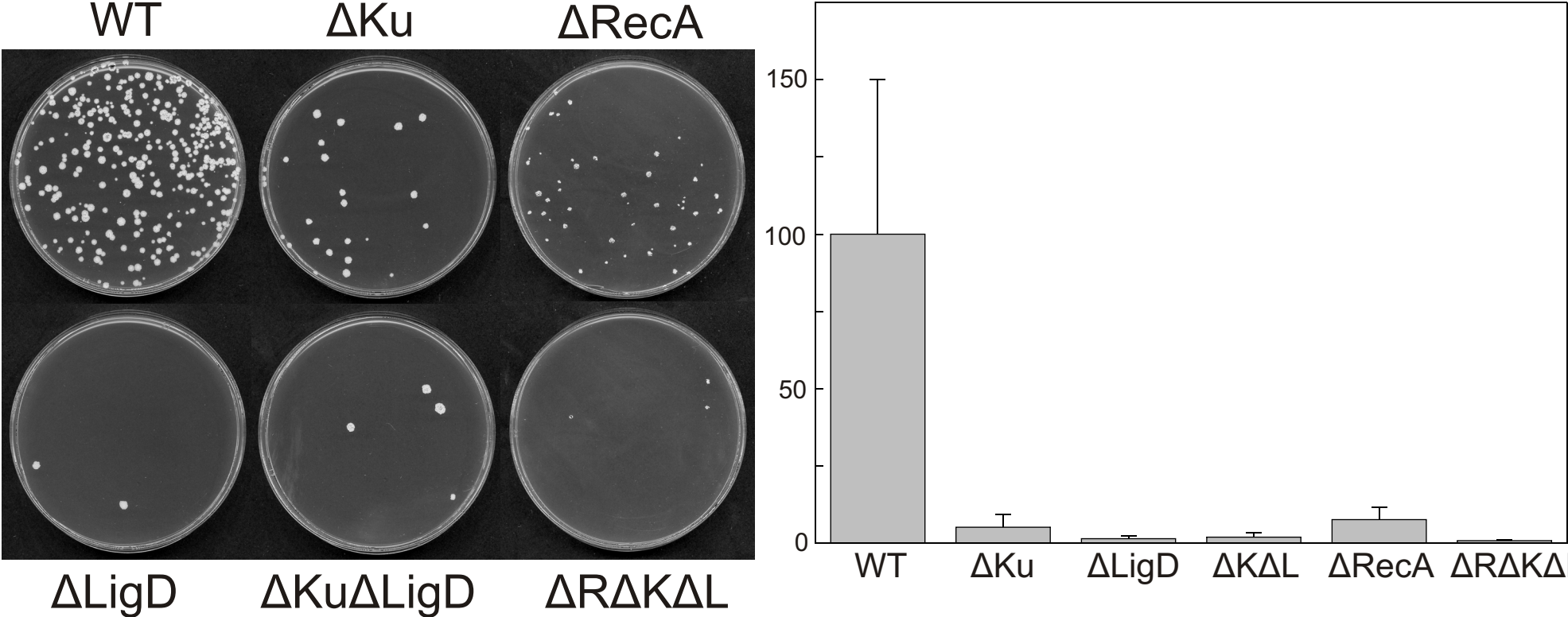


Figure 5



Supplementary information

Fig. S1 and S2- IR sensitivity of NHEJ-deficient strains of *M. smegmatis*.

Aliquots of wild-type and NHEJ-deficient (Δku , $\Delta ligD$ and $\Delta ku/ligD$) strains of *M. smegmatis* were taken from cultures at various points (A-D) in the growth-curve as indicated (right panel) and irradiated. Radiosensitivity was assessed following 0, 75, 225, 450 and 675 Gy of irradiation by spotting aliquots (10 μ l) of neat, 10x and 1000x dilutions of cells onto plates and incubating them at 37° C for three days (left panel).

Fig. S3 - IR sensitivity of $\Delta recA$ and $\Delta recA/ku/ligD$ strains of *M. smegmatis*.

Aliquots of $\Delta recA$ and $\Delta recA/ku/ligD$ strains of *M. smegmatis* were taken from cultures at various points in the growth-curve as indicated (right panel) and exposed to 0, 75, 225, and 450 Gy of IR. Aliquots (10 μ l) of neat, 10x and 1000x dilutions of the irradiated cells were spotted onto plates and incubated at 37° C for four days before colonies were examined (left panel).

